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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/699,076	10/27/2000	Nicos A. Petasis	06666/005002	9032

7590 02/25/2005

EXAMINER
SHIBUYA, MARK LANCE

ART UNIT	PAPER NUMBER
1639	

Scott C. Harris
FISH & RICHARDSON P.C.
4350 La Jolla Village Drive, Suite 500
San Diego, CA 92122

DATE MAILED: 02/25/2005

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	Application No.	Applicant(s)	
	09/699,076	PETASIS ET AL.	
	Examiner	Art Unit	
	Mark L. Shibuya	1639	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *08 September 2004*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *12,18-21,29 and 34-44* is/are pending in the application.

4a) Of the above claim(s) *34 and 40-42* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *12,18-21,29,35-39,43 and 44* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other: _____.

DETAILED ACTION

1. Claims 12, 18-21, 29 and 34-44 are pending. Claims 34 and 40-42 are withdrawn. Claims 12, 18-21, 29, 35-39, 43 and 44 are examined.

Continued Examination Under 37 CFR 1.114

2. A request for continued examination under 37 CFR 1.114, including the fee set forth in 37 CFR 1.17(e), was filed in this application after final rejection. Since this application is eligible for continued examination under 37 CFR 1.114, and the fee set forth in 37 CFR 1.17(e) has been timely paid, the finality of the previous Office action has been withdrawn pursuant to 37 CFR 1.114. Applicant's submission filed on 9/8/2004 has been entered.

Election/Restrictions

3. The Requirement for Election/Restrictions, as set forth and considered in Office actions mailed 10/10/2001, 3/27/2002, 10/17/2002 and 3/26/2003; and applicant's election of Group I (originally claim 12), without traverse, is maintained. Reservations regarding applicant's election of species, as discussed in the Office action, mailed 3/26/2003, are noted for the record.

4. Claims 34 and 40-42 remain withdrawn from further consideration as being drawn to non-elected species, there being no allowable generic or linking claim.

Withdrawn Claim Rejections

5. The rejection of claim 38 under 35 U.S.C. 112, first paragraph, for new matter, is withdrawn in view of applicant's amendment to the claim, filed 9/8/2004.

Maintained Claim Rejections

Maintained Claim Rejections - 35 USC § 102/103

The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

6. Claims 12, 18-21, 29, 35-39, 43 and 44 are rejected under 35 U.S.C. 102(b) as anticipated by or, in the alternative, under 35 U.S.C. 103(a) as obvious over Palfreyman et al., US 4,421,767. This rejection maintains the reasons of record, as set forth in the previous Office actions, mailed 11/12/2003 and 3/26/2003. The rejection of claim 44 is necessitated by applicant's addition of claim 44, filed 9/8/2004.

Response to Arguments

Applicant's arguments filed 9/8//2004 have been fully considered but they are not persuasive. Applicant argues that the reference of Palfreyman et al., US 4,421,767, does not disclose a combinatorial library, as defined in the instant application. Applicant argues that Palfreyman does not disclose a collection of compounds as instantly claimed, but instead discloses individual compounds represented as a Markush group. Applicant state that the compounds in the claimed library are related by location and by preparation. Applicant states that individual compounds disclosed as formula I, II or III in Palfreyman, does not disclose a collection as claimed in instant claim 12.

Applicant further argues that Palfreyman does not provide any teaching or suggestion to do that which applicant has done. Applicant argues that Palfreyman discloses individual compounds by formula I, II or III in a Markush format, and individual

compounds are not the same as a collection of compounds or a combinatorial library, as claimed.

Applicant further states that "the combinatorial library of the instant claims encompasses **mixtures of compounds and a collection of individual, pure compounds** prepared by the process recited in the claim. The claim is **not directed to individual compounds**." Applicant argues that the instant claim recites the feature that the plurality of compounds in the combinatorial library are prepared by the process recited in the claim. Again, applicant states that the library can be made as a mixture of compounds, or as a collection of individual pure compounds, depending on the methods used for identification of active compounds. Applicant states "[w]hen the library is made as a mixture of compounds, the compounds in the library are related by location as members of a common mixture having unique chemical and/or physical characteristics. When the library is made as a collection of individual pure compounds, the compounds are related by location as individual compounds distributed in a combinatorial array." Applicant states that the amendment to claim 12, now recites the limitation that plurality of compounds in the combinatorial library are prepared by the process recited in the claim.

Applicant's argument have been considered, but are not deemed persuasive. The examiner respectfully submits that the patent reference of Palfreyman discloses a collection of individual pure compounds. These compounds of Palfreyman are related in that they share a common core structure that confers a common property, as demonstrated by the common purpose to which they may be put, i.e., treatment of

mental depression. The reference of Palfreyman discloses anticipatory compounds I, II and III together, and contemplates treating patients with any one of compounds I, II or III. The examiner respectfully submits that, as such, Palfreyman discloses compounds I, II and III as an ensemble of molecules, sharing a method of synthesis, molecular core structure, properties, and purpose. Even although applicant argues that Palfreyman does not teach a "library" of compounds; however, the term is used quite broadly in the art to mean "any ensemble of molecules" (e.g., see Janda, K.D. "Tagged versus untagged libraries: Methods for the generation and screening of combinatorial chemical libraries", Proc. Nat'l. Acad. Sci., USA, November 1994, 91, 10779-10785, especially p. 10779, column 1, last sentence, "In its purest form, a combinatorial chemical library can be defined as any ensemble of molecules"). Therefore the examiner respectfully submits that the reference of Palfreyman et al. disclose a combinatorial library of compounds I, II and III.

Applicant's traversal of the instant rejection, as understood by the examiner, appears to argue that the compounds of a library must be related by location, as either in a mixture, or distributed in an array. These limitations are not required in the description of the term "combinatorial library", found in the specification as filed at p. 7, lines 14-36, although the specification teaches such mixtures or arrays may be preferred. For example, the specification, at p. 7, state that "it may be preferred to provide the library as a large mixture of compounds", or that "[l]arge combinatorial libraries may also be prepared by massively parallel synthesis of individual compounds, in which case compounds are typically identified by their position within an array."

Therefore, the examiner respectfully submits that these limitations are not found in the claims, and are precatory in the specification's description of combinatorial libraries. In response to applicant's argument that the references fail to show certain features of applicant's invention, it is noted that the features upon which applicant relies (i.e., libraries must have compounds as mixtures or arrays) are not recited in the rejected claim(s). Although the claims are interpreted in light of the specification, limitations from the specification are not read into the claims. See *In re Van Geuns*, 988 F.2d 1181, 26 USPQ2d 1057 (Fed. Cir. 1993).

Furthermore, it would have been obvious for one of ordinary skill in the art to have combined the compounds taught by Palfreyman, and thereby form a combinatorial library, because said compounds are used for a common purpose, i.e., the treatment of mental depression. See In re Kerhoven, 626 F.2d 846, 850, 205 USPQ 1069, 1072 (CCPA 1980) (stating: "It is prima facie obvious to combine two compositions each of which is taught by the prior art to be useful for the same purpose, in order to form a third composition to be used for the very same purpose.... [T]he idea of combining them flows logically from their having been individually taught in the prior art" [citations omitted]); and MPEP 2144.06.

New Claim Rejections

New Claim Rejections - 35 USC § 112

The following is a quotation of the second paragraph of 35 U.S.C. 112:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

7. Claims 12, 18-21, 29, 35-39, 43 and 44 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Claims 12 and 44 recite the limitation "the compound" in the last line. There is uncertain antecedent basis for this limitation in the claims.

New Claim Rejections - 35 USC § 102/103

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

This application currently names joint inventors. In considering patentability of the claims under 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of 35 U.S.C. 103(c) and potential 35 U.S.C. 102(e), (f) or (g) prior art under 35 U.S.C. 103(a).

8. Claims 12, 18-21, 29, 35-39, 43 and 44 are rejected under 35 U.S.C. 102(b) as anticipated by or, in the alternative, under 35 U.S.C. 103(a) as obvious over Kick et al., J. Med. Chem. 1995, 38, 1427-1430.

Kick et al. disclose collections of compounds (reading on the claimed "combinatorial library") that read directly on the instant formula **1**. See, compounds 7a, 7b and 7c in Kick et al., (p. 1428, para 3 and Figure 2). The compounds of the reference read on the claims where in R^1 is hydrogen, R^2 is carboxamido, R^3 and R^4 are independently hydrogen, and R^5 is heteroaryl.

In the alternative, although Kick et al., does not disclose that their collections of compounds (i.e. libraries) are made by the claimed process, the products of Kick et al. would appear to be the same as those recited by the instant claim, regardless of their method of manufacture. See MPEP 2113. Also note that many of the instant claims recite only process limitations and thus are non-limiting on the product.

The collection of compounds of Kick et al. (reading on the claimed "combinatorial library") meet all of the limitations of the claimed library except for the product-by-process limitations and would either anticipate or render obvious the claimed library. "[E]ven though product-by-process claims are limited by and defined by the process, determination of patentability is based on the product itself. The patentability of a product does not depend on its method of production. If the product in the product-by-process claim is the same as or obvious from a product of the prior art, the claim is unpatentable even though the prior product was made by a different process." In re Thorpe, 777 F.2d 695, 698, 227 USPQ 964, 966 (Fed. Cir. 1985).

The examiner respectfully submits that because the claim is drawn to a "combinatorial library", but this product is defined as a product-by-process, that any collection of compounds meeting the structural requirements of the instant claims reads

on this product. The process by which the claimed library is made does not appear to lend patentable weight to the claimed invention. One of ordinary skill would expect the library to be the same no matter how it was synthesized.

9. Claims 12, 18-21, 29, 35-39, 43 and 44 are rejected under 35 U.S.C. 102(b) as anticipated by or, in the alternative, under 35 U.S.C. 103(a) as obvious over Gordon et al., Bioorganic & Medicinal Chemistry Letters, 1995, Vol. 5, No. 1, pp. 47-50.

Gordon et al. disclose collections of compounds (reading on the claimed "combinatorial library") that read directly on alpha-amino acid derivatives of the formula depicted in claim 37. See, the compounds of the Tables found on p. 48 of Gordon and Scheme 2 on p. 49, and the text on said pages. Gordon et al. teach ten different alpha amino acids, which are then attached to resin bead, and mixed, providing a combinatorial library of alpha amino acids. These compounds of the reference read on the claims where the combinatorial library contains alpha amino acids.

In the alternative, although Gordon et al., does not disclose that their collections of compounds (i.e. libraries) are made by the claimed process, the products of Gordon et al. would appear to be the same as those recited by the instant claim, regardless of their method of manufacture. See MPEP 2113. Also note that many of the instant claims recite only process limitations and thus are non-limiting on the product.

The collection of compounds of Gordon et al. (reading on the claimed "combinatorial library") meet all of the limitations of the claimed library except for the product-by-process limitations and would either anticipate or render obvious the claimed

library. "[E]ven though product-by-process claims are limited by and defined by the process, determination of patentability is based on the product itself. The patentability of a product does not depend on its method of production. If the product in the product-by-process claim is the same as or obvious from a product of the prior art, the claim is unpatentable even though the prior product was made by a different process." In re Thorpe, 777 F.2d 695, 698, 227 USPQ 964, 966 (Fed. Cir. 1985).

The examiner respectfully submits that because the claim is drawn to a "combinatorial library", but this product is defined as a product-by-process, that any collection of compounds meeting the structural requirements of the instant claims reads on this product. The process by which the claimed library is made does not appear to lend patentable weight to the claimed invention. One of ordinary skill would expect the library to be the same no matter how it was synthesized.

Conclusion

10. Claims 12, 18-21, 29, 35-39, 43 and 44 are rejected. Claims 34 and 40-42 are withdrawn.

11. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Mark L. Shibuya whose telephone number is (571) 272-0806. The examiner can normally be reached on M-F, 8:30AM-5:00PM.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Andrew Wang can be reached on (571) 272-0811. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

Mark L. Shibuya
Examiner
Art Unit 1639

ms

Notice of References Cited	Application/Control No. 09/699,076	Applicant(s)/Patent Under Reexamination PETASIS ET AL.	
	Examiner Mark L. Shibuya	Art Unit 1639	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-			
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
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FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Janda, K.D. "Tagged versus untagged libraries: Methods for the generation and screening of combinatorial chemical libraries", Proc. Nat'l. Acad. Sci., USA, November 1994, 91, 10779-10785.
	V	Kick et al., J. Med. Chem. 1995, 38, 1427-1430.
	W	Gordon et al., Bioorganic & Medicinal Chemistry Letters, 1995, Vol. 5, No. 1, pp. 47-50.
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 10779–10785, November 1994

Review

Tagged versus untagged libraries: Methods for the generation and screening of combinatorial chemical libraries

Kim D. Janda

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ABSTRACT **Over the past two decades the pharmaceutical industry has been driven by the biological sciences. The discovery and description of the biological mechanisms that underlie disease states accompanied by an unraveling of these mechanisms has provided drug, and more recently biotechnological, companies with a barrage of new therapeutic targets. Paradoxically, as a result of such biological and biochemical advances, new sources of drug leads are in short supply. Considerable effort in trying to create potential drug candidates has led to the parturition of combinatorial chemical libraries. In this review I will examine some of the main technologies for generating and deducing active components from combinatorial libraries that have been segregated into two schools of thought: (*i*) the creation and decoding of combinatorial libraries by so-called tagged methodologies, and (*ii*) the production and deconvolution of chemical libraries by untagged protocols.**

The random screening of natural products from microbial fermentations, plant extracts, and marine sources for possible activity as therapeutic agents has been a rich source of new drug discoveries for years in the pharmaceutical sciences (1). However, with the advent of molecular biology and progress in crystallography and computational chemistry, "rational drug design" has found many advocates. The knowledge of the three-dimensional structure of receptors or enzymes and the manipulation of this information has lead to the development of a number of drug candidates including angiotensin converting enzyme (2, 3) and renin inhibitors (4–6). But even such "rational" success stories, initiated from first principles and based solely on structural information about the macromolecule alone, have not proved as fast or reliable as once claimed. In recent years there has been a renaissance in drug screening with an ingress of new technologies based on combinatorial chemical libraries (for review, see ref. 7). These methods expose many compounds to a target and allow the compounds that bind the target with the highest affinity to be filtered out from a pool of statistical sequences. In its purest form, a combinatorial chemical library can be defined as any ensemble of molecules, whereas the production and ultimately the methods of screening these combinatorial chemical libraries determine the "hit rate"—i.e., the success or failure of these collections of molecules (8).

Untagged Approaches to Generating Combinatorial Chemical Libraries

The two general procedural methodologies in formulating untagged combinatorial libraries are "mixture synthesis" and "portioning-mixing." Mixture synthesis is exactly what it sounds like, in that mixtures of chemical units are coupled to an activated support to produce chemical diversity (9–12). The advantage of such an approach is that combinatorial libraries of vast complexity, in theory, should be accessible. The problem of achieving such diversity is that product dispersal is strongly influenced by the relative kinetic rates of each competing chemical unit being coupled. Deviating from reactions where the kinetic constants for the addition of each individual component are unknown could be disastrous to the product distribution. Portioning-mixing (13–15), or what has come to be known as combinatorial library split synthesis, is a two-step operation based on a divide–couple and recombine procedure. The essentials of this strategy are that a polymeric support is divided into equal portions for coupling to modular units [such units have typically been amino acids; however, in principle, any chemical moiety (say X) could be appended to the support]. The matrices are combined in a single vessel for washing and/or deprotection and then divided again for the next coupling. Repeating this protocol for a total of n cycles can produce a stochastic collection of up to X^n different molecules. More important, this strategy allows for an equal distribution of the coupled chemical units and for uniform couplings to occur.

Various approaches for generating and screening untagged combinatorial chemical libraries have been developed. Each has advantages and disadvantages in its efforts to create chemical diversity. Several of the most prominent will be considered below.

Mimotope Strategy

Geysen and coworkers (11, 16, 17) have presented an amide linkage strategy wherein peptides can be synthesized in a reusable format. The peptides are synthesized on polymer matrices, "pins," positioned within a microtiter 96-well plate. In this approach, natural L-amino acids, as well as unnatural D-amino acids (we will term either enantiomer X), can be incorporated into the library; thus, X^n peptides are conceivable. However, to manage such large numbers with this pin format dipeptide units are initially held constant, whereas the rest of the sequence is formed from randomly incorporated amino acids. To synthesize a hexapeptide library in this regime requires $NNX_{[3]}X_{[4]}NN$, where now $X_{[3]}$ and $X_{[4]}$* are defined amino acids at positions 3 and 4, respectively, and N represents positions where residues are randomly incorporated by using reaction mixtures containing natural or unnatural amino acids. If a primary set is made using an alphabet of 20 amino acids, then the size of this set will be 400 (20 × 20 preparations), each of which is now a mixture of peptides theoretically consisting of 160,000 different peptides. The derivatization level will be at ≈100 nmol per pin or an average of $\approx 4 \times 10^{11}$ copies of each of the individual peptides.

Examining this primary set of 400 peptide mixtures for binding with an antibody or receptor allows identification of the optimum dipeptide sequence for $X_{[3]}X_{[4]}$. A second screen (40 peptide mixtures) $NNX_{[3]}X_{[4]}D_{[5]}N$ and $ND_{[2]}X_{[3]}X_{[4]}NN$, wherein the amino acids $X_{[3]}X_{[4]}$ are fixed and D equates to positions where single amino acids are to be incorporated, allows resolution or absolute identification of additional residues. This cycle of synthesis and screening is reiterated until the entire hexapeptide sequence is optimized for binding to the target of interest.

Originally this mimotope strategy was used to determine discontinuous epitopes with a protein antigen (12). Recently, its scope has been expanded to include the screening of other relevant receptors

*Subscripts in brackets throughout text represent residue positions rather than multiples.

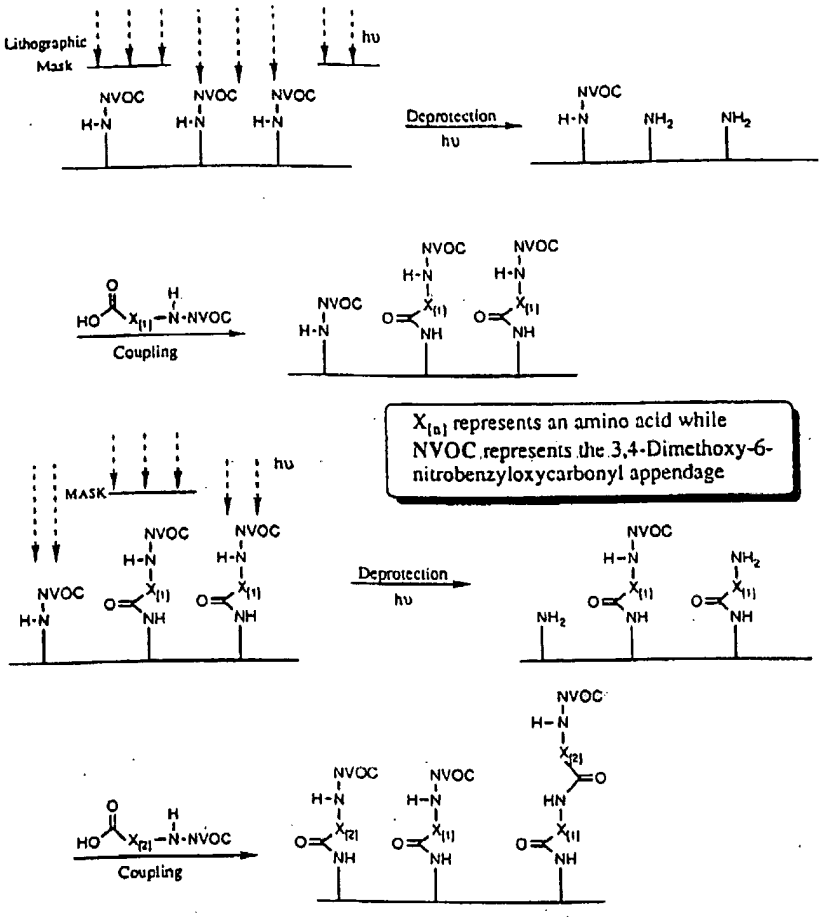

FIG. 1. Scheme of how peptide combinatorial libraries can be constructed using the light-directed, spatially addressable parallel chemical synthetic approach developed by Foder *et al.* (19).

(18). The positive aspects of the mimotope scheme are that modular synthetic strategies are ultimately used in secondary, tertiary, etc. . . . screens. The library diversity can also be increased by applying α-disubstituted and β-amino acids to the process.

Light-Directed, Spatially Addressable Parallel Synthesis of Combinatorial Libraries

Fodor and his colleagues at Affymax (19) have demonstrated a technology that intertwines solid-phase synthesis with photolithography in the preparation of peptide and oligonucleotide combinatorial libraries (Fig. 1). Their general procedure was to derivatize an aliphatic amino-terminated matrix with the light-sensitive amino-protecting group 3,4-dimethoxy-6-nitrobenzyloxycarbonyl (NVOC). Photolysis of the surface removes the protecting group and thereby activates the area for further synthesis. The synthetic scaling process that ensues depends on the photolithographic masking pattern used. Thus, after photolysis (deprotection) the entire surface is exposed to the next NVOC-protected amino acid or oligonucleotide, wherein coupling of either unit occurs only in the regions exposed to light. The procedure is repeated until all the building blocks are coupled to the support. The pattern of masks and the sequence of reactants define the products and their locations. In other words, the identity of the sequence and its location are known.

Screening for activity within these synthesized libraries is done via a fluorescent reporter-group assay, wherein a fluorescently tagged receptor or enzyme is allowed to interact with the library. The fluorescent intensity at each site will depend on the affinity of the receptor for the compound, the concentration of the receptor, and the number and density of the sites resulting in the binding event.

The strengths of this approach are that high-density arrays of chemical compounds can be created in a very small area. A 10-step binary synthesis with amino acids results in the formation of 1024 peptides in 1.6 cm^2. Furthermore, the chemistry is not limited to just peptide and oligonucleotide synthetic strategies. As evidence, the Schultz group (20) has extended the Fodor technology to an oligocarbamate library (Fig. 2).

One-Bead, One-Peptide Solid-Support Technology

A one-bead, one-peptide combinatorial library using split synthesis was pioneered in a collaborative effort by workers at the University of Arizona and the Selectide Corporation (21). Their approach is defined by a noncleavable linker moiety that attaches the peptide unit to the bead. Library screening is accomplished by using receptors conjugated to fluorescein or an enzyme; either entity distinguishes an association event between relevant library members and an acceptor molecule from the rest of the population. Individual library member beads that are stained by this process are removed and analyzed by Edman microsequencing to deduce the sequence of the corresponding peptide ligand.

Using this methodology, the Tucson group has synthesized a 19-amino acid (less cysteine) pentapeptide library (2,476,099 members). The library was screened against a monoclonal antibody known to bind the β-endorphin epitope (YGGFL) with a K_d of 17.5 nM. The

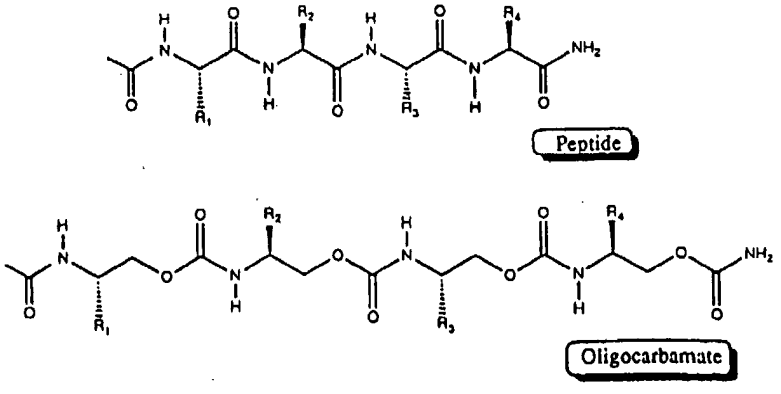

FIG. 2. A peptide and the corresponding oligocarbamate. R represents any standard amino acid side chain.

antibody 3E7 conjugated to alkaline phosphatase retrieved six new pentapeptide-binding sequences with K_d values from 15 to 8780 nM.

The power of this one-bead, one-peptide split synthesis concept lies in the fact that any solid-phase chemistry can theoretically be applied and investigated in a receptor-based and/or enzyme assay. However, this advantage also leads to drawbacks, one of which is that libraries must be screened attached to the support. Subsequent work has been directed at using multicleavable linker appendages to allow for screening of soluble peptide mixtures (22).

Dual-Defined, Positional Scanning, and Robotics Library Technology

Houghten *et al.* (23–25), using what they termed a "dual-defined iterative" methodology, have assembled soluble combinatorial peptide libraries via split synthesis. In the seminal paper (23) a dual-defined hexapeptide library containing 18 amino acids was constructed as follows: Four cycles using solid-phase peptide synthesis and "portioning-mixing" provided 104,976 protected tetrapeptide resin sequences (18^4). This partial library of NNNN-resins (where N is a randomized amino acid position) was divided into 324 aliquots, so that the synthesis of the next two positions $X_{[1]}X_{[2]}$ NNNN-resin could be defined (X is an amino acid position that is defined; the bracketed subscript indicates residue position); upon deprotection and cleavage from these respective resins, a now complete soluble hexapeptide library of >34 million members was obtained. These 324 pools are assayed, and positive results for the first two residues (say $A_{[1]}B_{[2]}$) were noted. Next, 18 new libraries were synthesized with the formula $A_{[1]}B_{[2]}X_{[3]}$NNN, one for each amino acid at position 3, and tested to define $X_{[3]}$. The process is repeated until all positions are defined. Essentially, this methodology is an iterated search process that consists of making the library in a number of segregated pools, finding the active pool that defines the entity for the position on the molecule, and then repeating the process until the active component has been identified. A similar iterative process called SURF (synthetic unrandomization of randomized fragments) was used by ISIS Pharmaceuticals (Carlsbad, CA) for an oligonucleotide library (26).

A virtue of this dual-defined iterative technology is that the multiplicity of components decreases with each step, so that an enrichment process occurs, and because molecules can be assayed in solution, it permits functional, as well as binding, assays. An application of this iterative strategy includes the discovery of antimicrobial peptides with activities against Gram-negative and Gram-positive bacteria (23, 24).

The positional scanning format is again based on soluble combinatorial libraries; however, its proof of concept has been shown to be viable using mixture synthesis (27, 28); now for the same hexapeptide library of 18 amino acids, six different libraries (i.e., 108 positional scanning sublibraries) of the general formula $X_{[1]}N_{[2]}N_{[3]}N_{[4]}N_{[5]}N_{[6]}$, $N_{[1]}X_{[2]}N_{[3]}N_{[4]}N_{[5]}N_{[6]}$, $N_{[1]}N_{[2]}X_{[3]}N_{[4]}N_{[5]}N_{[6]}$, $N_{[1]}N_{[2]}N_{[3]}X_{[4]}N_{[5]}N_{[6]}$, $N_{[1]}N_{[2]}N_{[3]}N_{[4]}X_{[5]}N_{[6]}$, and $N_{[1]}N_{[2]}N_{[3]}N_{[4]}N_{[5]}X_{[6]}$ must be synthesized and assayed. This technology defines the preferred residue at each position of the sequence. The technology also alleviates the unwieldy iterative synthesis and selection steps required in the dual-defined methodology. However, this strategy, unlike the dual-defined methodology, is not endowed with an enrichment process or a progressive improvement of the signal-to-noise ratio.

The Chiron group has developed a fully automated peptide synthesizer that allows combinatorial peptide libraries to be created in the split-synthesis format (29, 30). The instrument consists of an array of reaction vessels, solenoid valves, and a Zymark robot that is computer controlled. The use of this instrument was shown by synthesizing a 361-member decapeptide library. Through competitive ELISA and an affinity-selection methodology decapeptide library members were identified that bound an anti-gp120 monoclonal antibody.

A Recursive Deconvolution Strategy

The final "nontagged" combinatorial library methodology to be described is that of my group (31). The essence of our method is to build and hold a set of partially synthesized combinatorial libraries. A formal example of our method is shown in Fig. 3 for a library of degree 3, made from an alphabet of three components, A, B, and C. In our process we define three channels of synthesis, and each involves only the addition of a single component. Initiating the process requires the making of three pools, in which A, B, and C are added to a matrix adapted with a linker for coupling of the components. A portion of this library is set aside and labeled as partial library p(1). This portion or fraction to be saved and catalogued is the inverse of the degree of the library in the first step. Hence, in the example presented, a degree of three, one-third of each pool is saved and labeled; for subsequent steps the amount saved and catalogued is the inverse of degree minus one. The remaining material is combined and separated into three portions, each channel is loaded, and A, B, and C are attached as before. Again, an aliquot of this library is set aside as partial library p(2), which now is three pools made up of $N_{[1]}A_{[2]}$, $N_{[1]}B_{[2]}$, and $N_{[1]}C_{[2]}$. The remainder is again pooled and split, and the

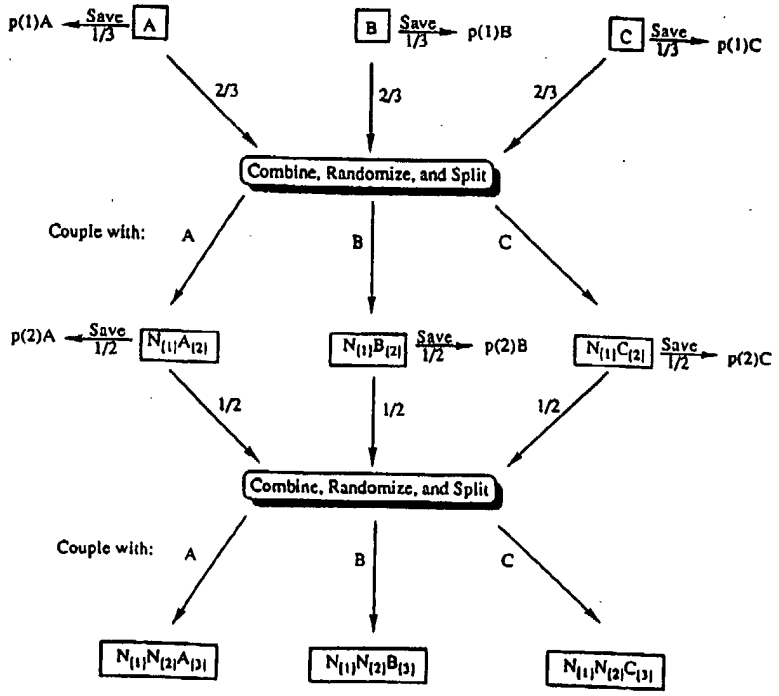

FIG. 3. General scheme of how a chemical combinatorial library using the concept of recursive deconvolution is synthesized and cataloged. Each final pool contains 9 molecules; there is a total of 27 unique molecules in this example. Subscripts

third step of addition is carried out to give the final library $N_{[1]}N_{[2]}A_{[3]}$, $N_{[1]}N_{[2]}B_{[3]}$, and $N_{[1]}N_{[2]}C_{[3]}$.

Screening against a receptor, ligand, or even an enzyme and then determining the most active library member(s) is done simply by examining the final three pools first and proceeding backward to the partial libraries saved. Thus, for the example presented in Fig. 3, we have three pools, $N_{[1]}N_{[2]}X_{[3]}$ (X is A, B, or C), nine compounds in each pool (i.e., a total of 27 different compounds), which are tested by an appropriate assay, and the active pool is determined. Suppose $N_{[1]}N_{[2]}B_{[3]}$ from the final library shown in Fig. 3 is positive. We then go back to library p(2) and add B to an aliquot of each of the three pools, P(2)A, P(2)B, P(2)C, to give three new libraries of the general formula $N_{[1]}X_{[2]}B_{[3]}$. These three new libraries now contain only nine components, so a 3-fold enrichment has been achieved. Again, after testing, suppose pool $N_{[1]}A_{[2]}B_{[3]}$ is active. We proceed to partial library P(1) and add A to each followed by B to give three new pools with the structure $X_{[1]}A_{[2]}B_{[3]}$, which can be tested to find $X_{[1]}$. Again, a 3-fold enrichment has been achieved; the structure is synthesized, and the sequence is deduced.

We have examined this recursive deconvolution in a peptide combinatorial library that was tailored to contain pentapeptide sequences that display binding to the commercially available anti-β-endorphin monoclonal antibody 3E7. In the final analysis, the native epitope YGGFL was found to be the most extensive binder; however, other weaker binders were also deduced through this strategy (31).

There are a number of advantages in using this recursive deconvolution strategy. (*i*) Split synthesis, a rather cumbersome process, need only be done once for each combinatorial library made. In stark contrast is the dual-defined method, which requires numerous split synthetic operations. (*ii*) The deconvolution process recursively defines the synthesis of the active component, so that in the last cycle, the active compound is synthesized. In addition, this methodology allows the deduction of alternative active members, as each deconvolution pathway can be followed either in parallel or successively. (*iii*) Any chemistry is applicable with this technology, which, as we will see, can be problematic in generation of encoded combinatorial libraries.

Tagged Methodologies to Generating Combinatorial Chemical Libraries

Evident from the discussion presented is that there are three critical aspects in any combinatorial library: (*i*) The chemical units that go into the library, (*ii*) the technique for generating the library, and (*iii*) identification of library members that interact with the biological target of interest. Although these three points have been addressed in several of the "untagged" combinatorial library protocols described, limitations do exist. In this next section alternative strategies will be provided, which are termed "tagged" combinatorial libraries.

Phage Technology

Arguably, one of the most powerful tag technologies is that of strictly biological origin. The general concept is one in which a library of peptides is presented on the surface of a bacteriophage such that each phage displays a unique peptide and contains within its genome the corresponding DNA sequence (32–34). In detail, foreign DNA can be inserted into the minor coat protein locus (gene III) of filamentous phage to create fusion phage that express these corresponding peptides at the N terminus of the absorption protein (pIII), which is displayed on the phage surface (Fig. 4). The diversity of displayed peptides is generated by cloning randomly synthesized oligonucleotides that are inserted into a specific region of gene III. These phage libraries encoding peptide units can possess as many as 10^9 unique sequences, which can be screened for binding to any type of immobilized receptor in a selection process known as "panning." This method uses an affinity capture technique to select peptide display phage that bind to the receptor of interest. Selected phages are amplified by infecting *E. coli*, whereas each cycle of panning and amplification enriches certain peptide display phage sequences that bind most tightly to the receptor molecule. After the panning process, DNA from the isolated phage is sequenced, and the peptide responsible for binding is elucidated.

The application of phage technology to the binding of receptors (antibodies) has been demonstrated by several groups (35, 36), including Smith's group (34), who have shown that an epitope hexapeptide library ($\approx 10^7$ members) displayed within the pIII protein could provide individual members that bound to two different antibodies that were specifically made to the DFEKI peptide unit found on the surface of myohemerythrin. In a library designed by Devlin *et al.* (37) phage display epitope libraries with 15, rather than 6, amino acids were made. This approach not only increases the effective size of the library, it also allows the possibility of the display of discontinuous epitopes.

The strengths of phage technology are that large combinatorial libraries of peptide fragments can be generated very quickly and efficiently. Of greater importance is that these peptide libraries are physically linked to their own encoding tag (DNA), which allows for user-friendly amplification, enrichment, and decoding of pertinent binding sequences. At first glance, this methodology seems very appealing; however, a glaring weakness inherent in this technology and other "genetic" library methodologies is that the overall diversity of the chemical units that can be applied within these systems is finite. Exploitation of these libraries for drug discovery may thus be limited.

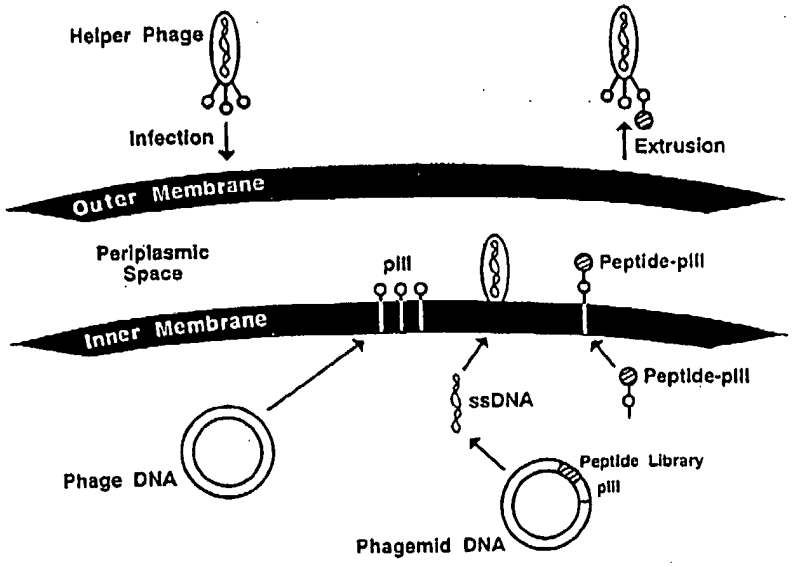

FIG. 4. Scheme illustrating the proposed pathway for peptide display on filamentous bacteriophage. Helper phage infects *Escherichia coli* cells harboring phagemid DNA that contains the genes for the peptide libraries. Helper phage DNA is used to express native pIII, whereas phagemid DNA is used to express peptide–pIII. Single-stranded (ss) phagemid DNA is packaged into phage particles through the aid of helper phage-encoded proteins.

Peptides-on-Plasmids

An alternative to phage technology, which also relies on a biological tack is a genetic combinatorial library approach termed "peptides-on-plasmids." What Schatz and his coworkers at Affymax (38) have done is establish another efficient methodology for a physical connection between a peptide and nucleic acid that encodes for it. In their procedure, a library of peptides is constructed so that the genetic material encoding them is linked through the DNA-binding activity of the lac repressor protein. The random peptides are fused to the C terminus of the repressor by cloning degenerate oligonucleotides at the 3′ of the repressor gene (lac I) present on a plasmid. This plasmid also has lac repressor-binding sites, so the fusions bind the same plasmid that encodes them. Assay for peptide-receptor binding in the Schatz approach is quite similar to the panning process in phage methodology; however, now cell lysis is used to liberate the peptide–lac-plasmid complexes that are screened repeatedly for affinity enrichment.

A proof of principle of this work came from the construction of a random dodecamer library. This library was used to probe for potential peptide members that could bind to IgG D32.39, a specific hybridoma to the dynorphin B epitope. As expected, a consensus sequence was discovered that corresponded to similar binding studies of phage libraries (35). More recently, this peptides-on-plasmid approach has been extended to a more challenging system—the discovery of new substrates for *E. coli* biotin haloenzyme synthetase. The results were that smaller peptide units (13-residue sequences) could be used as substrates (39).

Two features distinguish the peptides-on-plasmid approach from phage technology. (*i*) Random peptides are displayed with a free C terminus. (*ii*) The repressor fusions are cytoplasmic, whereas phage fusions are periplasmic. However, as with phage technology this methodology is restricted in its repertoire of chemical building blocks to oligopeptide units.

Peptide Coded Libraries

In an attempt to address the diversity problem inherent in genetic libraries while still retaining the advantages of a tagging unit, the Chiron Corporation group devised a chemical approach with peptides as the encoding unit (40). The chemical combinatorial strategy uses resin-splitting peptide synthesis to alternately synthesize a "binding" strand and a "coding" strand. An orthogonal protecting-group scheme is also used to allow for the parallel synthesis of both chemical units on the resin shown in Fig. 5.

The isolation of receptor-binding ligands from a tagged library of this type can be done by affinity-selection or bead-staining techniques. The identification of the binding sequence can be determined by Edman sequencing. However, when using such sequencing technology, the binding strand, if of peptide composition, must be made nonsequenceable. In an analogous fashion, the Selectide Corporation group (41) has also described a peptidic coding approach. In their report, protecting-group schemes are used that allow an assay for library receptor binding, either on the matrix or in a soluble format.

The most desirable feature of this type of peptide encoding strategy is that it grants a potential for alternative chemical units other than the natural amino acids (or nucleotides) to be incorporated into the binding strand. However, this methodology does not allow for enrichment by serial selection, the cornerstone upon which genetic library selection methods are founded.

Electrophoric Polyhalobenzene Coded Libraries

Another solution to the chemical coding of combinatorial libraries has been disclosed recently by Still and coworkers (42). In their approach, a combinatorial peptide library attached to beads was assembled using the split synthesis method, while simultaneously being indexed by what they refer to as electrophoric tagging. In their scheme, a series of aromatic electrophores varying in hydrocarbon chain length (Fig. 6) are used as tagging units. Tagging is done by an alternating synthetic process that coincides with the addition of each library binding unit; however, the tagging process here does not require sequential connection. To further simplify the entire scheme, the tags are used in a binary code to record the addition of each building block and, thus, the reaction history of each bead.

Screening of the library is done by a reporter-group assay (*vide infra*), and identified beads with affinity to the receptor are individually picked out by a micropipetter. Use of the *o*-nitrobenzylcarbonate moiety on the linker portion of each coding unit allows release of tag alcohols through photolysis, which can be analyzed by electron capture gas chromatography and identified by the binary synthesis code. Borchardt and Still (43) have advanced this technology to the synthesis of a combinatorial N-acylated tripeptide library using both D- and L-amino acids. This library was used to probe the binding requirements of a synthetic receptor. The outcome of this study was that it allowed new types of host–guest interactions to be observed that might not have been discovered by conventional studies.

A clear advantage of the Still coding technology is that numerous types of chemical processes are amenable to it. Thus, the potential to create highly diversified chemical libraries is well within its purview. The tagging methodology is elegant, as no cosynthesis is required. Again, however, this methodology, like the peptide-tagging technology, does not allow for amplification or enrichment to operate.

Encoded Combinatorial Libraries

If the breadth and versatility of chemical synthesis could be linked to the power of genetics, a potentially more diverse approach to tagged combinatorial libraries could be achieved. A conceptual method to bridge the gap between these two different realms and apply it to combinatorial libraries was advanced by Brenner and Lerner (44) and termed encoded combinatorial chemistry. In their theory, to carry out encoded combinatorial chemistry (Fig. 7) two alternating parallel combinatorial syntheses must be done so that a genetic tag is chemically linked to the chemical structure being synthesized. Thus, addition of a chemical unit to a matrix is followed by addition of an oligonucleotide sequence that codes the chemical unit appended. As with all chem-

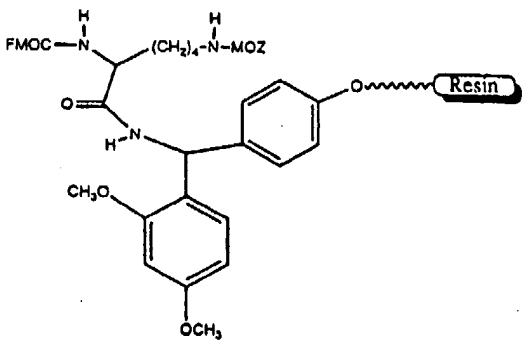

FIG. 5. Resin and bifunctional linker unit containing orthogonal protecting groups to allow for alternating synthesis of the binding and coding strands in peptide coded libraries. FMOC, 9-fluorenylmethyloxycarbonyl functionality; MOZ, 4-methoxybenzyloxycarbonyl.

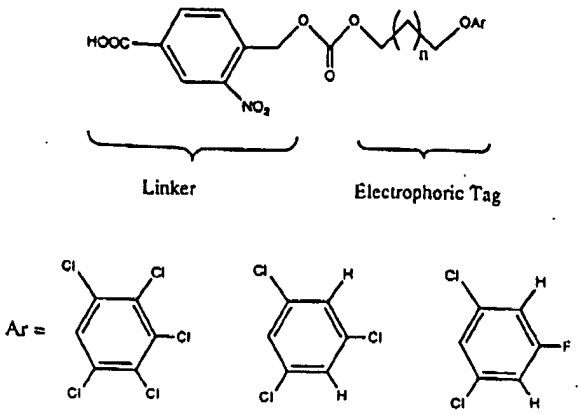

FIG. 6. Tagging units used to create binary synthesis code for electrophoric polyhalobenzene coded libraries.

ical tagging technologies, split synthesis is applied in making the library. However, where this encoded approach differs from other coded synthetic methodologies is in the selection process between library members and receptors. Now active library molecules are affinity-selected to a receptor, and amplified copies of their retrogenetic tags are obtained via PCR. DNA strands that are amplified can be used to enrich for a subset of the library by hybridization with the matching tags, and this type of panning process can be repeated with this subset. Thus, serial enrichment is achieved by a process of purification. Ultimately, the DNA of binding members can be decoded to provide the chemical history and, hence, the structure of the binding unit.

The general principles of this technology are straightforward. The chemical manipulations to create such libraries can be complex. If one just considers synthesizing a chemical library of peptides, new linker technologies, protecting-group schemes, and, moreover, synthetic protocols must be contemplated and devised. At the time when this encoded combinatorial chemical library approach was disclosed, the alternating parallel synthesis of peptides and oligonucleotides had yet to be described. My group recently demonstrated the synthetic methods needed for implementation of this type of mixed chemical synthesis (45, 46). The gist of our tack was to make solid-phase peptide and oligonucleotide synthesis compatible. We accomplished this through the use of 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry for the peptide portion of the molecule, whereas a methyl phosphate scheme was engaged for the oligonucleotide-encoding unit. Following our lead, similar synthetic methodologies applied to beads have also been published by the group at Affymax (47). In their program, an oligonucleotide-encoded D, L peptide library was made on a 10-μm support. Screening the library for binding to an epitope on dynorphin B provided a number of peptide members after amplification and decoding.

The fervor that encoded combinatorial libraries brings to the chemical library field is evident, as it exploits the best of both worlds—chemical diversity and the power of genetics. The main constraints in using this technology come from its strength—namely, the tagging unit. Oligonucleotides can be chemically labile and incompatible with certain synthetic procedures. However, even with these limitations, the elegance of this approach is hard to surpass.

Concluding Remarks and Future Directions

Within a very short time span, a large body of work has accumulated within the field of combinatorial chemical libraries. As with any new scientific endeavor, different paths of research are explored in trying to advance the subject. I have presented what has come to be a philosophical divide within the scientific community on combinatorial libraries: the

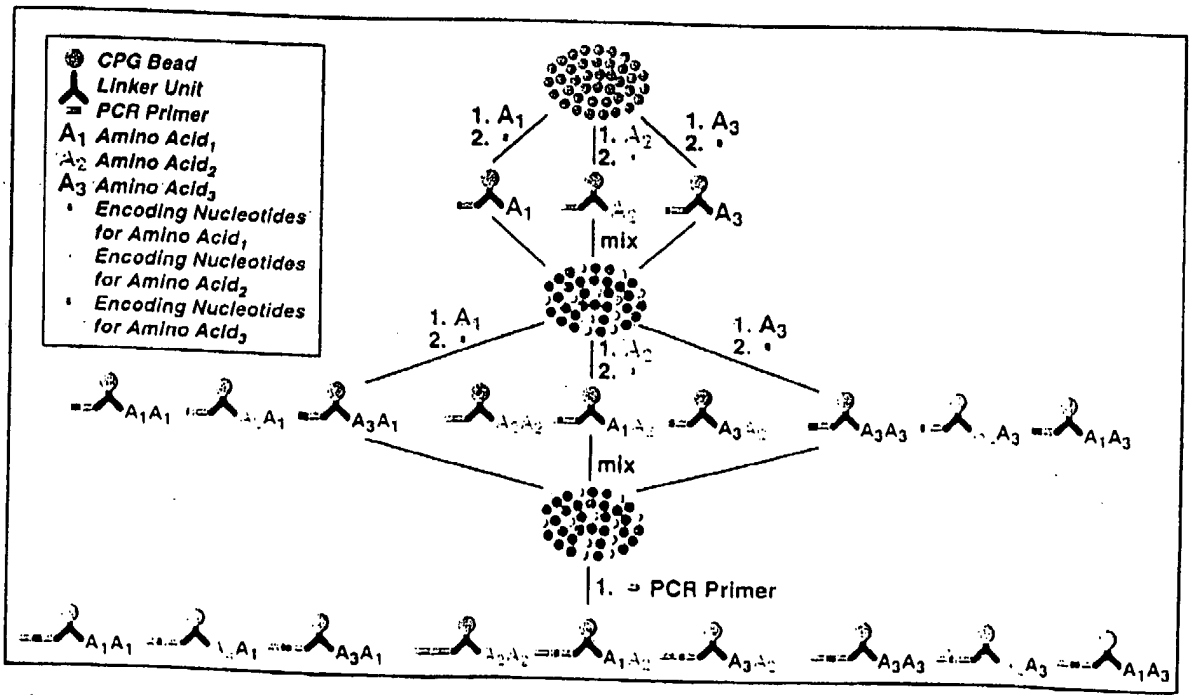

FIG. 7. Scheme that describes the general format used in obtaining encoded combinatorial chemical libraries. CPG, controlled pore glass. Subscripts denote residue position. The figure is reproduced with permission from ref. 45 (copyright (1993) American Chemical Society).

notion of tagging the combinatorial entities or leaving them as untagged components. Clearly, strong arguments can be made in defense of either technology, but ultimately the deciding factor as to what methodology will be engaged depends on user need. Addressable libraries or ones procured by split synthesis for an iterative or recursive deconvolution approach have the potential to create the most diverse combinatorial libraries. In contrast, coded libraries, specifically those of genetic origin, allow for the screening of receptors that are rare and/or of limited concentration.

Currently, most available combinatorial libraries (tagged or untagged) are of either peptide or nucleotide origin. It appears that the next wave of combinatorial research will be directed at the design of libraries that are devoid of the repetitive backbone linkage found within peptides or nucleotides. It is here that structural/stereoelectronic variation and unconstrained motifs will be allowed to expand to unparalleled combinatorial chemical diversity. Already important advances in this area by Bunin and Ellman (48) and Hobbs Dewitt *et al.* (49) have provided us with a glimpse of possible developments.

Finally, it should be noted that combinatorial chemical libraries can provide us with another paradigm for drug discovery and development. Although the field is still young, the methods/technologies for generating and screening these libraries is already quite diverse and is becoming increasingly more sophisticated. The quest for the future is to find whether combinatorial chemical libraries can provide us with more than just "lead" sources for drug discovery. As long as the essence of drug discovery is to find quick and cost-effective new drug candidates, combinatorial chemical libraries provide a unique and ever changing source of chemical diversity.

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Communications to the Editor

Expedient Method for the Solid-Phase Synthesis of Aspartic Acid Protease Inhibitors Directed toward the Generation of Libraries

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Introduction. Aspartic acid proteases are a widely distributed family of enzymes that play important roles in fungi, plants, vertebrates, and retroviruses.[1] The aspartic acid proteases (characterized by having two aspartic acid residues in the active site) catalyze the hydrolysis of amide bonds with specificity for peptide bonds located between large hydrophobic residues. A number of aspartic acid proteases are important pharmaceutical targets, including renin,[2] cathepsin D,[3] the human immunodeficiency virus (HIV) protease,[4,5] human t-cell leukemia virus type 1 (HTLV-1) protease,[6] and *candida albicans* aspartic acid protease.[7]

Potent inhibitors of these enzymes can be readily accessed by the incorporation of an isostere that mimics the geometry of the tetrahedral intermediate in place of the scissile bond of the peptide substrate.[2b,8] Unfortunately, these inhibitors have limited therapeutic utility, due to the poor oral availability and/or short-circulating half-lives that result from their peptidic nature. For this reason, there has been a great deal of work toward the development of aspartic acid protease inhibitors that display *nonpeptide* functionality about the isostere of the tetrahedral intermediate.[2,4] In order to generate a therapeutically useful inhibitor the compound must have both high affinity and favorable pharmokinetic properties. The combination of these two requirements render an *a priori* design of inhibitor structure based on the peptide substrate very challenging. Therefore, the identification of potent and bioavailable inhibitors has required the time-consuming synthesis and evaluation of a large number of different nonpeptidic compounds.

Herein we report a general and high-yielding solid-phase method for the rapid display of *nonpeptide* functionality about molecules incorporating the (hydroxyethyl)amine and (hydroxyethyl)urea isosteres.[9] Of the possible isosteres upon which to construct a library of potential nonpeptide inhibitors, the (hydroxyethyl)-amine and (hydroxyethyl)urea isosteres were selected for two reasons. First, several orally available HIV-1 protease inhibitors that incorporate these isosteres have been identified (Figure 1),[4] including compounds that are currently in clinical trials for the treatment of HIV infection. Second, we believed that solid-phase methods could be developed to display a wide range of diverse functionality about these isosteres.

Initially we chose to display functionality from scaffold 1,[10] which provides access to known HIV-1 protease inhibitors. The scaffold was first coupled to dihydropyran functionalized polystyrene support by employing

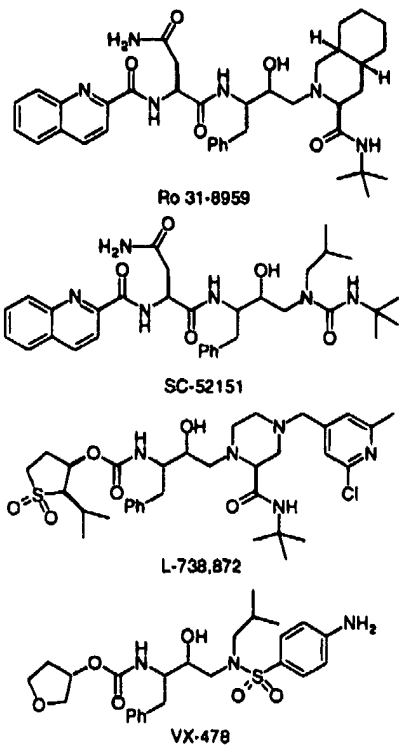
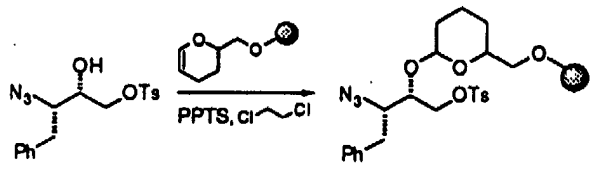


Figure 1.

Scheme 1



pyridinium *p*-toluenesulfonate in 1,2-dichloroethane (Scheme 1).[11] The reaction progress was qualitatively monitored by IR by following the appearance of the azide stretch. The exact loading level of the resin was based on the mass balance of recovered alcohol **1**, which was obtained by subjecting a portion of the resin **2** to cleavage by 95:5 trifluoroacetic acid (TFA)/water.[12]

The synthesis was initiated by displacement of the primary tosyl alcohol with either functionalized or unfunctionalized primary or secondary amines, including amines found in known HIV-1 protease inhibitors (Scheme 2, **3a–h** and **4i**). After coupling of the primary amines, the resulting secondary amine products **3a–g** can be converted to ureas by reaction with isocyanates (**4a–f**) or by stepwise treatment with triphosgene followed by amine addition (**4g**).[13,14] The ability to employ either a preformed isocyanate or the stepwise procedure to synthesize the ureas provides ready incorporation of functionality from both commercially available isocyanates and the even larger pool of commercially available amines. Notably, substituted ureas have been employed successfully at this site in a number of

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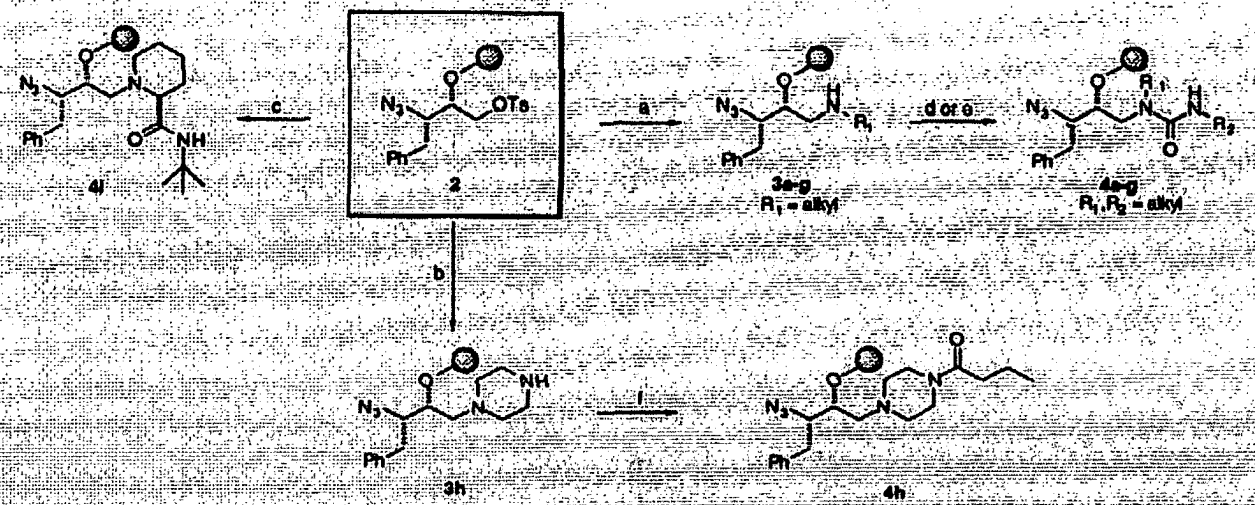
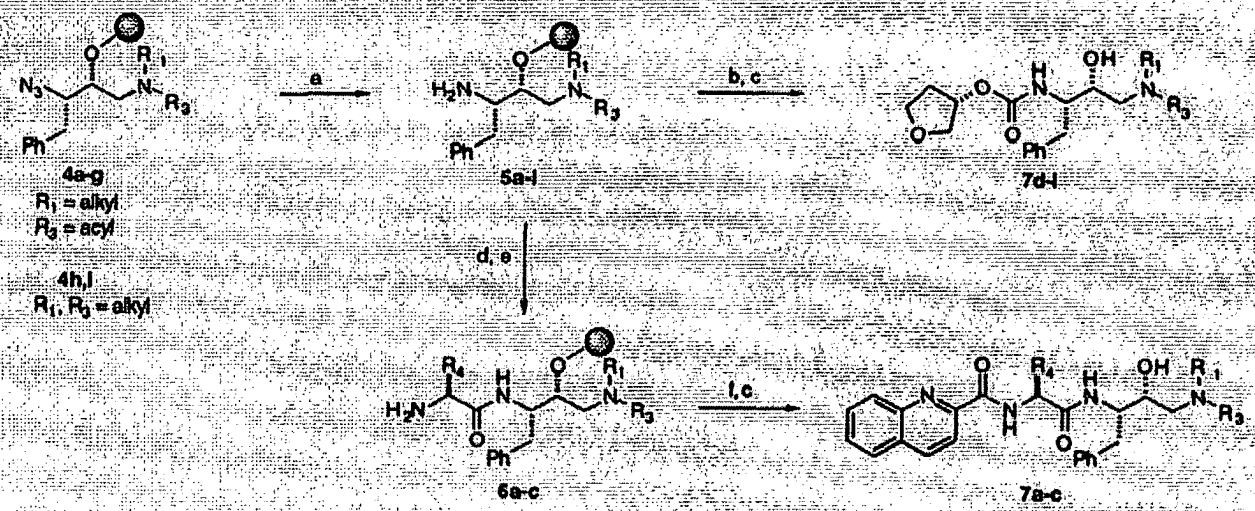
Scheme 2[a]



[a] (a) R_1NH_2, NMP, 80 °C; (b) piperazine, NMP, 80 °C; (c) *N-tert*-butyl-L-pipecolinamide, NMP, 95 °C; (d) R_2NCO, $ClCH_2CH_2Cl$; (e) (i) $OC(OCCl_3)_2$, Et_3N, cat. DMAP, THF, (ii) 4-(3-aminopropyl)morpholine, THF; (f) butyryl chloride, *i*-Pr_2EtN, CH_2Cl_2.

Scheme 3[a]



[a] (a) $SnCl_2$:HSPh:Et_3N (1:4:5), THF, (b) 3(*S*)-tetrahydrofuranylsuccinimidyl, *i*-Pr_2EtN, CH_2Cl_2; (c) 95:5 TFA/H_2O; (d) Fmoc amino acid, *PyBOP*, *HOBt*, *i*-Pr_2EtN (3 equiv), DMF; (e) 20% piperidine in DMF; (f) pentafluorophenyl ester of quinaldic acid, HOBt, Et_3N, DMF.

aspartic acid protease inhibitors.[4b,15] In addition, acyl chlorides can be employed to provide amides; for example, the piperazine derivative **3h** was acylated with butyryl chloride to provide **4h** (Scheme 2).

The synthesis about the P_1 site of the inhibitor was initiated by reduction of the azide using thiophenol/Et_3N/$SnCl_2$ (4:5:1) as described by Bartra and coworkers (Scheme 3).[16] The reduction was relatively rapid (<4 h at room temperature), and the reaction progress was easily monitored by IR by following the disappearance of the azide stretch. Although a number of alternative methods are available for reducing azides to amines, most of these methods are heterogeneous in nature, are slow, and/or require protic solvents that do not effectively solvate the polystyrene resin.

The resulting primary amine (**5**) can then be acylated to provide carbamate or amide products that can be further derivatized. For example, the coupling of **5a** with *N*-Fmoc-Asn(Trt)-OH under PyBOP/HOBt coupling conditions[17] was followed by removal of the Fmoc protecting group with 20% piperidine in DMF to provide **6a** (Scheme 3). Subsequently, the free amine was coupled with the pentafluorophenyl ester of quinaldic acid.[18,19] The concomitant removal of the trityl protecting group and the cleavage of the material from the solid support with 95:5 TFA/water for 20 min[12] provided the HIV-1 protease inhibitor **7a** (Figure 2), developed by Monsanto,[4b] in 85% overall yield for the six-step process. To demonstrate the versatility of the method, compounds **7b** and **7c** were prepared by incorporating the sterically hindered amino acid, valine, and the functionalized amino acid, tyrosine, in place of asparagine in 83% and 74% overall yields, respectively. Alternatively, reaction of amines **5d–l** with the activated *N*-succinimidyl carbonate of 3(*S*)-hydroxytetrahydrofuran provides carbamates **7d–l**.[20] The tetrahydrofuran urethane has been shown to bind tightly to the S_2 region of HIV-1 protease.[4c,21] Cleavage of the material from the solid support with 95:5 TFA/water for 20 min[12] provided analytically pure derivatives **7d–l** after chromatography in 47–86% overall yield based on the initial loading of alcohol **1**.

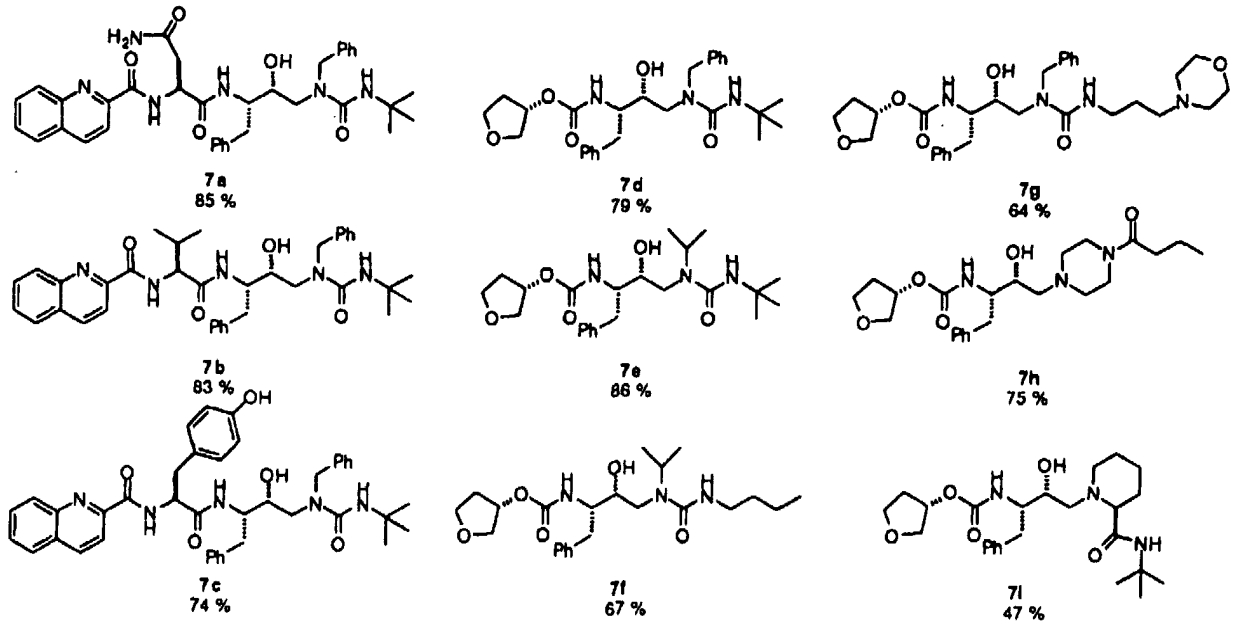


Figure 2. (Hydroxyethyl)amine and (hydroxyethyl)urea derivatives synthesized on solid support. Yields of analytically pure material after chromatography were determined from mass balance and were based upon the initial loading of alcohol 1. Elemental analyses were all within ±0.4% of theoretical value.

In summary, we have been able to obtain good yields (47–86%) of molecules incorporating the (hydroxyethyl)-amine and (hydroxyethyl)urea isosteres after four to six chemical transformations on solid support. In addition, we have been able to incorporate many of the functional groups and structures that are present in known inhibitors of HIV-1 protease and renin, thereby demonstrating the generality of the synthesis sequence. Employing the described synthesis method, the simultaneous synthesis of a library of potential aspartic acid protease inhibitors is in progress, as is the evaluation of the library against a number of aspartic acid protease targets.[22]

Acknowledgment. The NSF, Burroughs Wellcome Fund, the A. P. Sloan Foundation, American Cyanamid, Upjohn, Hoffman La Roche, Eli Lilly, and Tularik provided financial support for this work. We thank Bruce Szczepankiewicz for his helpful discussions about triphosgene-mediated urea synthesis from secondary amines. Structure searches were performed using the MDL Drug Data Report (MDDR), MDL Information Systems, Inc., 14600 Catalina Street, San Leandro, CA 94577.

Supplementary Material Available: Experimental details for the synthesis and characterization of compounds **7a–i** (5 pages). Ordering information is given on any current masthead page.

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REDUCTIVE ALKYLATION ON A SOLID PHASE: SYNTHESIS OF A PIPERAZINEDIONE COMBINATORIAL LIBRARY

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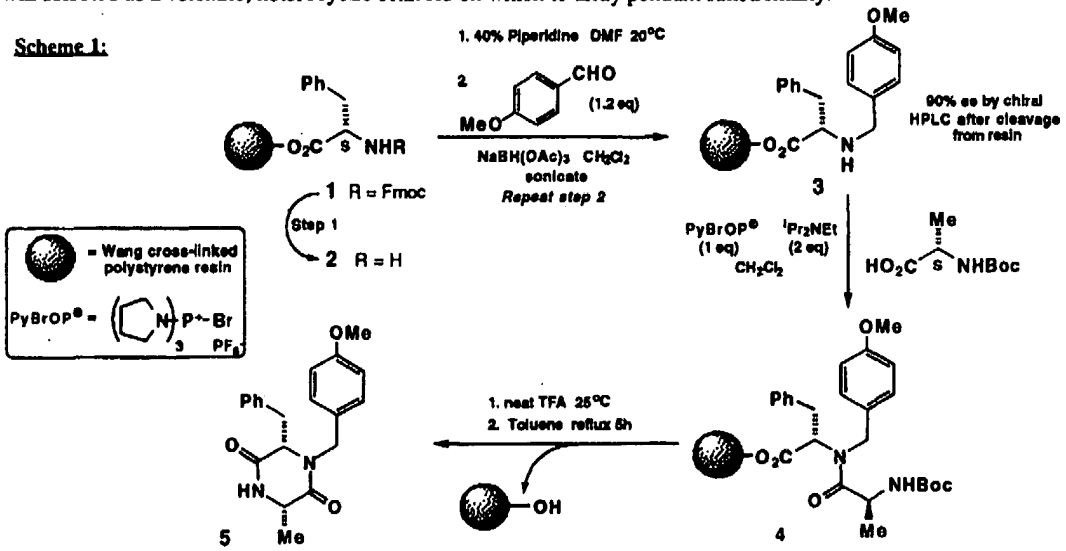
Abstract: *The synthesis of a prototype trisubstituted piperazinedione combinatorial library of 1,000 compounds has been achieved from three precursor sets - two sets of ten α-amino acids and one set of ten aldehydes. A sodium triacetoxyborohydride-mediated reductive alkylation was crucial to the success of the multi-step synthesis on resin. This protocol represents a new method to augment compound files rapidly with novel heterocyclic entities for high-speed screening.*

Combinatorial synthesis of libraries containing linear peptide and non-peptide structures is widely recognised as a valuable new tool for lead discovery in the search for new therapeutics. Of the methods for achieving the synthesis of large numbers of library components, one of the most flexible is the resin-based mix and split technology, first described by Furka and co-workers in 1988[1] and subsequently developed further by several other groups.[2,3] The speed and convenience of screening compound mixtures is a powerful aspect of this technology. In general, this technique has been restricted to peptide-type coupling reactions,[2] although solid-phase chemistry for the assembly of some heterocycles as individual compounds is now becoming established.[4] We describe here the solid-phase synthesis of a prototype combinatorial library of 1,000 piperazinediones (diketopiperazines or DKPs) **5**, each containing three centres of molecular diversity. The rigid DKP template was selected as a versatile, heterocyclic scaffold on which to array pendant functionality.

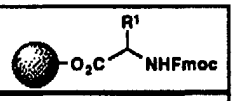
Scheme 1:



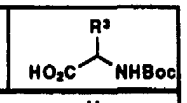
Scheme 1 contains the synthetic protocol for a representative DKP derived from Fmoc-Phe-Wang resin **1**, Boc-Ala and *p*-methoxybenzaldehyde.

The reductive alkylation of peptides on MBH[5] and Merrifield[6] resins using sodium cyanoborohydride in acidified DMF is a well-established process but pH sensitivity and potential reagent toxicity made this an unattractive option for the present work. We have instead used an efficient sodium triacetoxyborohydride-facilitated alkylation of amino acids such as **2** on Wang resin by a range of aldehydes, affording an array of novel, resin-bound secondary amines including **3**. Yields for the general process **2**→**3** are typically 85-95%, although partial racemisation of some homochiral amino acids (notably Phe as shown) was observed. This reductive alkylation has proved compatible with a wide range of natural and synthetic α-amino acids and is tolerant of almost any aldehyde, although in a potential 'worst case' combination of a hindered amino acid (e.g. Val, see later) and an electronically deactivated aldehyde, isolated yields of ~20% were obtained, the balance of recovered material being the unreacted amino acid. Aliphatic aldehydes also gave minor but significant amounts of *bis* alkylation (1-10%), generating tertiary amines. Coupling of a relatively unreactive secondary amine (eg **3**) to a further Boc-protected amino acid proved to be a greater chemical challenge. After examination of a range of activated ester coupling techniques, this transformation was best achieved in our hands using a PyBrOP mediated double-coupling protocol, generating an amide (eg **4**). A double coupling was found to be essential to achieve consistent yields of >90%. The optimal method of assembling target DKPs such as **5** employed a TFA-mediated Boc deprotection and concomitant resin cleavage followed by a short reflux of the evaporated filtrate in toluene to induce cyclisation. Without the thermolysis in toluene, no DKP formation was observed in any of our work. In the specific case given in ***Scheme 1***, the overall yield for synthesis of the pure DKP **5** was 42% based on resin-bound amino acid with trace amounts of bis-alkylated Phe recovered as the only identifiable by-product. Chiral HPLC analysis[7] of a sample of the reductive alkylation product **3** after resin cleavage indicated slight racemisation, presumably occurring prior to reduction of the intermediate imine.

The monomeric components used for the prototype piperazinedione library are given in the ***Tables*** below:

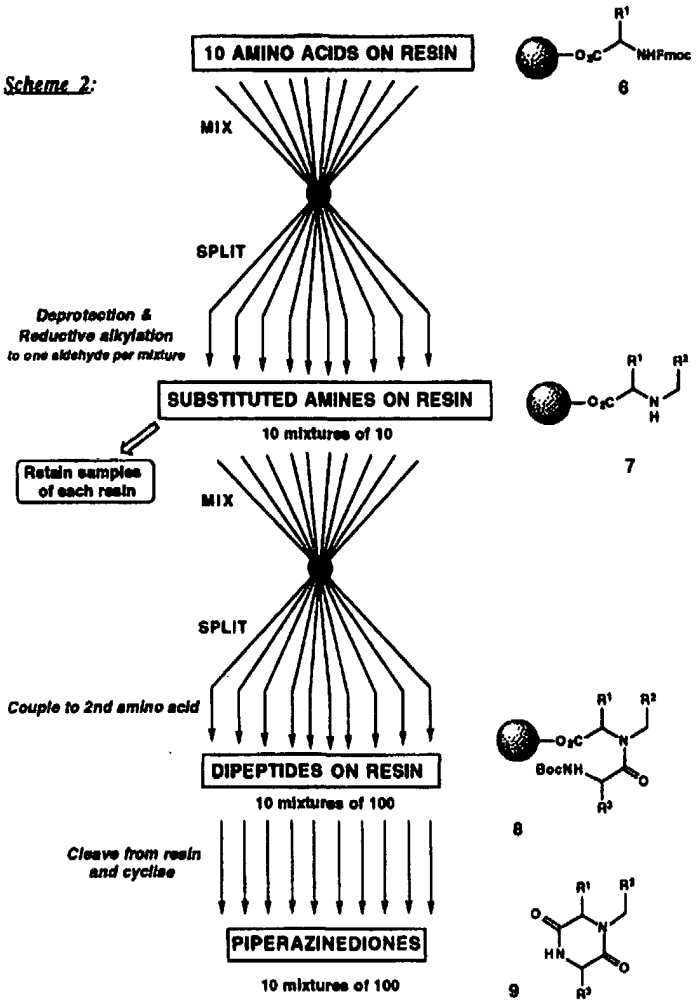
Fmoc amino acids	
Gly	H
Ala	Me
Val	Me, Me
Leu	Me, Me
D-Phe	
Ser(O^{t}Bu)	$OCMe_3$
D-Met	SMe
Lys(Boc)	NHBoc
Arg(pmc)	NHpmc, NH
Asn(trt)	$NHCPh_3$, O

Aldehydes (R^2-CHO)	R^2
	n-C_8H_{17}
	MeS
	NC
	Cl
	MeO, MeO, MeO

Boc amino acids	
Gly	H
Ala	Me
Leu	Me, Me
Phe	
Nle	Me
Met	SMe
Cys(SMe)	SMe
D-Ala	Me
Pro	HO_2C, N, Boc
Tyr	OH

To assemble a prototype combinatorial library of 1,000 piperazinediones, the protocol in ***Scheme 2*** was used:[8]

Scheme 2:



Ten Fmoc-protected amino acids **6** on Wang resin were intimately mixed and then distributed as equivalent mixtures into ten reactor vessels. Each mixture was deprotected using piperidine and reductively alkylated with a single aldehyde. Samples of the resultant secondary amines on resin **7** (~5% by wt) were retained for analysis and subsequent synthetic follow-up of any biologically active mixtures. The remaining resins were mixed again and redistributed into ten further mixtures. Each mixture was coupled to a single amino acid, then the resultant products **8** were cleaved and cyclised as described earlier for the single DKP synthesis to afford ten mixtures, each containing 100 DKPs **9** - overall constructing **a total of 1,000 compounds from an initial pool of 27 contributing units**.

The comprehensive analysis of multicomponent mixtures presents a significant problem. Clearly, some of the 1,000 components may be under-represented as a consequence of two or more consecutive low yields. The aim of our analysis has been to fully validate the chemistry, then characterise intermediate stages in the library synthesis to generate confidence that most (>95%) of the expected components are likely to be present. For the DKP library, this was achieved by assembly and full characterisation of a range of individual, pure DKPs including representative syntheses of potential 'worst-case' products such as **9** (R^1=^{i}Pr, R^3=^{i}Bu, R^2=3,4,5-trimethoxyphenyl - 24% overall). For the intermediate ten-component mixtures of secondary amino acids (prepared by TFA digestion of **7**), HPLC-MS combined with MS-MS was used to unambiguously confirm the presence of 96 of the expected 100 components (ref 9 contains the experimental protocol used for this analysis). Spectroscopic methods are still under development for the effective analysis of 100-component mixtures.

Acknowledgements: We are indebted to Dr. Adrian Wright, Mr. George Perkins and Mr. Keith Burton of the Physical Sciences group at Sandwich for HPLC and MS analytical data.

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3. For two useful recent surveys of library methods, see:
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   b) Pavia, M.R.; Sawyer, T.K.; Moos, W.H. *Bioorg. Med. Chem. Lett.*, **1993**, *3*, 387 and subsequent articles in that issue.
4. For example: Bunin, B.A.; Ellman, J.A. *J. Am. Chem. Soc.*, **1992**, *114*, 10997 (benzodiazepinones) and DeWitt, S.H.; Kiely, J.S.; Stankovic, C.J.; Schroeder, M.C.; Reynolds Cody, D.M.; Pavia, M.R. *Proc. Natl. Acad. Sci. USA*, **1993**, *90*, 6909 (hydantoins).
5. Coy, D.H.; Hocart, S.J.; Sasaki, Y. *Tetrahedron*, **1988**, *44*, 835. Hocart, S.J.; Nekola, M.V.; Coy, D.H. *J. Med. Chem.*, **1987**, *30*, 739.
6. Tourwé, D.; Piron, J.; Defreyn, P.; Van Binst, G. *Tetrahedron Lett.*, **1993**, *34*, 5499.
7. HPLC analytical separation achieved using a reverse phase ES-OVM 4.6mm x 16cm column, eluting 97:3 phosphate buffer (pH6.6): MeCN at 1ml/min. UV detection at 270nM.
8. Experimental details for the library assembly are as follows:
   Deprotection: Fmoc amino acids on resin (Wang cross-linked polystyrene, 0.2mmol each; weights varied with resin loading density) were combined, intimately mixed and stirred in 40%v/v piperidine/DMF (40ml) for 20 minutes. The resin was drained, washed (5xDMF), dried and the entire deprotection repeated. ***Reductive alkylation:*** This dry resin was evenly distributed into each of ten round-bottomed flasks and each suspended in CH_2Cl_2 (0.5ml). To each was added one of the ten aldehydes (0.24mmol) in CH_2Cl_2 (0.5ml). Each flask was sonicated in an ultrasound bath for 20 minutes followed by addition of a pre-sonicated solution of sodium triacetoxyborohydride (0.28mmol) in dichloromethane (0.5ml). All reactors were sonicated for five minutes then stirred vigorously for 16h. Each resin was filtered, washed (H_2O, aqueous $NaHCO_3$, H_2O, THF: 3x2ml each), dried and the reductive alkylation procedure repeated. 10% by weight of each dry resin-bound secondary amine intermediate was retained for future analysis and iterative follow-up. ***Coupling:*** All remaining resin was intimately mixed and evenly distributed to each of ten flasks. Each was suspended in CH_2Cl_2 (3ml) and treated with a solution of one of the ten Boc-amino acid components (0.2mmol) in CH_2Cl_2 (0.5ml and DMF, if necessary, to achieve complete solubility), followed by a solution of PyBrOP (0.2mmol) in CH_2Cl_2 (0.5ml) and finally diisopropylamine (0.4mmol). Each reaction was stirred for 24h then filtered, washed (DMF, H_2O, THF), dried and the coupling process repeated. ***Resin Cleavage:*** Each resin was suspended in trifluoroacetic acid (TFA, 1ml) for 3h with occasional agitation, then filtered and washed with CH_2Cl_2 (5ml). Each filtrate was concentrated, the residue dissolved in toluene and concentrated once more to remove any residual TFA. ***DKP cyclisation:*** The residues were dissolved in toluene (10ml) and stirred under reflux for 5h, then evaporated to dryness and the resultant compound mixtures dissolved in DMSO prior to automated screening.
9. **LC/MS analysis.** Putative ten-component mixture samples were analysed by capillary LC-MS using a 150 x 0.32mm capillary column containing hypersil ODS 5μm packing, operating at a flow rate of 3 μl/min. Separations were achieved using a water/acetonitrile/trifluoroacetic acid gradient, increasing from 25% to 50% acetonitrile over 30 min. The mobile phase contained 10% glycerol as a matrix for the Continuous Flow Liquid Secondary Ionisation Mass Spectrometry (CF-LSIMS) interface. All of the eluent was transferred directly to the CF-LSIMS interface on a Kratos Concept-1S mass spectrometer scanning from 725 to 150 amu in 3 seconds. **Direct MS analysis** of the mixtures without prior separation was achieved by chemical ionisation on a VG Trio-3 triple quadrupole instrument using ammonia as the reagent gas. Component structures were confirmed by daughter ion MS/MS using argon as the collision gas.

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